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Identification and Origin of Hemoglobin in a Gymnophallid Metacercaria (Trematoda:Digenea), a Symbiote in the Marine Polychaete *Amphitrite ornata* (Annelida:Terebellidae)

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Abstract. Unencysted metacercariae belonging to the family Gymnophallidae (Trematoda:Digenea) were isolated from the metanephridial sacs of the common marine worm *Amphitrite ornata* (Polychaeta:Terebellidae). These metacercariae possess an intracellular red pigment that has characteristic absorption spectra for oxygenated, deoxygenated, and carbon monoxide derivatives of hemoglobin when exposed to oxygen, nitrogen, and carbon monoxide, respectively. The hemoglobin, found in all metacercaria tissues, has an oxygen half-saturation (P_{50}) value = 1.1 (S.D. = 0.3) mm Hg at 20°C and pH 7.0. The pigment shows cooperative oxygen binding with a Hill coefficient of 2.2 (S.D. = 0.2) and exhibits a significant Bohr effect between pH 6.8 and 7.4. The hemoglobin has a high molecular weight fraction (~ 2.5–3×10^6 daltons) and a 16,000 dalton MW fraction. HPLC ion exchange chromatography shows four distinct protein components. The host possesses both vascular extracellular hemoglobin (erythrocruorin) and coelomic cell hemoglobin. The metacercariae ingest host coelomic cell hemoglobin and probably vascular hemoglobin; however, metacercaria tissue hemoglobin is functionally and biochemically distinct from both host hemoglobins, suggesting an independent origin.

Introduction

Metacercariae in the family Gymnophallidae (Trematoda:Digenea) occur primarily in the extrapallial space of bivalve and gastropod molluscs (Cable, 1953; Stunkard and Uzmann, 1962). Exceptions recorded in the literature for three species in the genus *Gymnophallus* and one species in the genus *Parvatrema* include the use of polychaetous annelids as the second intermediate host (Oglesby, 1965; Margolis, 1971, 1973; Nikitina, 1976; Bartoli, 1981). Kyle and Noblet (1985) found unencysted gymnophallid metacercariae on the external body wall and tentacles of the marine polychaete *Amphitrite ornata* (Annelida:Terebellidae). Subsequently these larvae were found in the metanephridia of *A. ornata* (Burden and Noblet, 1987). These distome larvae range from 0.05 to 0.8 mm in diameter and from 0.1 to 1.5 mm in length. The metacercariae are a diffuse reddish-brown and many have bright red ceca. The identity of these larvae is unknown.

Although many trematodes are pink or red, only a few species have been shown to possess hemoglobin. The presence of hemoglobin in parasites was reviewed by Lee and Smith (1965). Since then only a few additional trematodes have been examined for hemoglobin (Lutz and Siddiqi, 1967; Cain, 1969a).

Based primarily on spectral evidence, Lee and Smith (1965) concluded that parasite hemoglobins were different from host hemoglobins. Lutz and Siddiqi (1967) determined by electrophoresis that the hemoglobin of *Fasciola gigantica* is distinct from its host hemoglobin. Cain (1969b) determined through radio-tracer experiments that *Fasciolopsis buski* synthesizes the globin moiety of its hemoglobin, but does not synthesize heme. Hemoglobins from two species of trematodes (*Fasciolopsis buski* and *Dicrocoelium dendriticum*) have been well-characterized biochemically (Cain, 1969a, c; Tuchschmid *et al.*, 1978; Di Iorio *et al.*, 1985; Smit *et al.*, 1986), but very little is known about hemoglobin function in any trema-

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tode parasite. Heme proteins, as identified by positive benzidine tests, occur in a few trematode larvae (Cain, 1969a), but the benzidine test does not distinguish between hemoglobins and other heme proteins or peroxidases.

The reddish-brown pigment of the gymnophallid metacercariae in *Amphitrite ornata* shows characteristic absorption spectra for hemoglobin, and the pigment exhibits reversible spectral changes upon alternate exposure to O_2 and N_2. Spectral examination of intact living animals showed hemoglobin to be present in every location of the metacercaria body.

Amphitrite ornata possesses both a vascular extracellular hemoglobin and a coelomic cell hemoglobin (Mangum *et al.*, 1975). Host vascular hemoglobin, sometimes termed erythrocruorin, is a high molecular weight molecule (MW = 3×10^6 daltons) found free in solution (hereafter referred to as HMW hemoglobin), while host coelomic cell hemoglobin is a monomer (MW = 16,000 daltons) (Weber *et al.*, 1977).

The bright red coloration of the ceca of metacercariae recently removed from the polychaete host suggests that the metacercariae may ingest hemoglobin from their host. Consequently, several possibilities were considered for the origin of hemoglobin in metacercaria body tissues. Metacercaria hemoglobin may represent one or both host hemoglobins or slightly modified versions of these pigments, or it may represent a unique pigment or pigments produced in the larval trematode from products derived from digestion of host hemoglobins and other substances (*i.e. de novo* synthesis). The present study was undertaken to characterize the physical and chemical properties of the hemoglobin found in tissues of these metacercariae and to compare metacercaria hemoglobin to host hemoglobins.

Materials and Methods

Specimens of *Amphitrite ornata* were collected from an intertidal mud flat at Garden City, South Carolina, and transported to Clemson University where they were placed in culture dishes in seawater. They were maintained at 16°C and the seawater was changed every one to two days. Metacercariae were removed from the metanephridia of *Amphitrite* and placed in fresh filtered (0.45 μm) seawater. They were used immediately for spectrophotometric analyses or were starved for 24 to 48 hours for use in biochemical analyses. Starvation ensured that host hemoglobins would not be present in the guts of the metacercariae. Following the starvation period, the animals were placed in a small amount of fresh filtered seawater and stored at −80°C.

Absorption spectra of host and metacercaria hemoglobins were obtained using a single beam microspectrophotometer as described by Colacino and Kraus (1984). Oxygen equilibrium measurements were made using a modification of a standard two wavelength technique (Rossi-Fanelli and Antonini, 1958). Animals were placed in 50 m*M* Tris buffered seawater between two teflon membranes (12.5 μm thick) in a specially designed gas slide within which gas tension and temperature were controlled (Colacino and Kraus, 1984).

Metacercariae used for histological sectioning were relaxed in three milliliters of seawater to which two to three small crystals of MS-222 (3-aminobenzoic acid ethyl ester) were added. Following relaxation, animals were fixed in 2.5% gluteraldehyde in Millonig's phosphate buffer (=960 mOsm). Postfixation was in 1% osmium tetroxide in the same buffer followed by alcohol dehydration and embedment in Polybed 812 resin. Whole animal cross sections (1 μm) stained with Methylene Blue and Azure B stain were photographed using a Zeiss Photomicroscope I. Ultrathin sections stained with lead citrate and uranyl acetate were examined on a Phillips 300 transmission electron microscope.

Metacercaria hemoglobin samples were prepared by sonicating thawed animals. Cell debris was removed by centrifugation at 16,000 × *g* for 2 minutes at 4°C. The resulting clear supernatant was placed on ice until used.

Amphitrite HMW hemoglobin was collected by first rinsing whole animals in 0.45 μm filtered seawater and blotting the animals on filter paper. The animals were then placed on parafilm and a gill filament was cut. The HMW vascular hemoglobin was pipetted into a centrifuge tube and placed on ice. The HMW hemoglobin was centrifuged at 16,000 × *g* for 2 min at 4°C to remove any cell debris or particulate contaminants and the clear red supernatant was diluted 1:1 with 0.1 *M* Tris buffered seawater, pH 7.4. Samples were placed on ice for immediate use, or were stored at −80°C for later analyses.

Amphitrite coelomocytes were collected by first cleaning the animals as described above, then making a small incision into the coelomic cavity. Coelomic fluid containing coelomocytes was pipetted into a centrifuge tube. The cells were washed two times with 0.05 *M* Tris buffered seawater, pH 7.4, then placed on ice. For chromatography, cells were lysed by freezing at −80°C. Cell debris was removed by centrifugation at 16,000 × *g* for 5 min at 4°C. The resulting clear red supernatant was placed on ice until used.

The pH of *Amphitrite* coelomic fluid was measured during collection of coelomic fluid. After the incision was made, a miniature pH electrode was placed into the coelomic fluid as it leaked from the opening. This method may introduce errors in the measured pH due to loss of carbon dioxide. After collection of the fluid, the sample was aerated and the pH remeasured. The pH was always more basic following aeration. Thus, errors in our

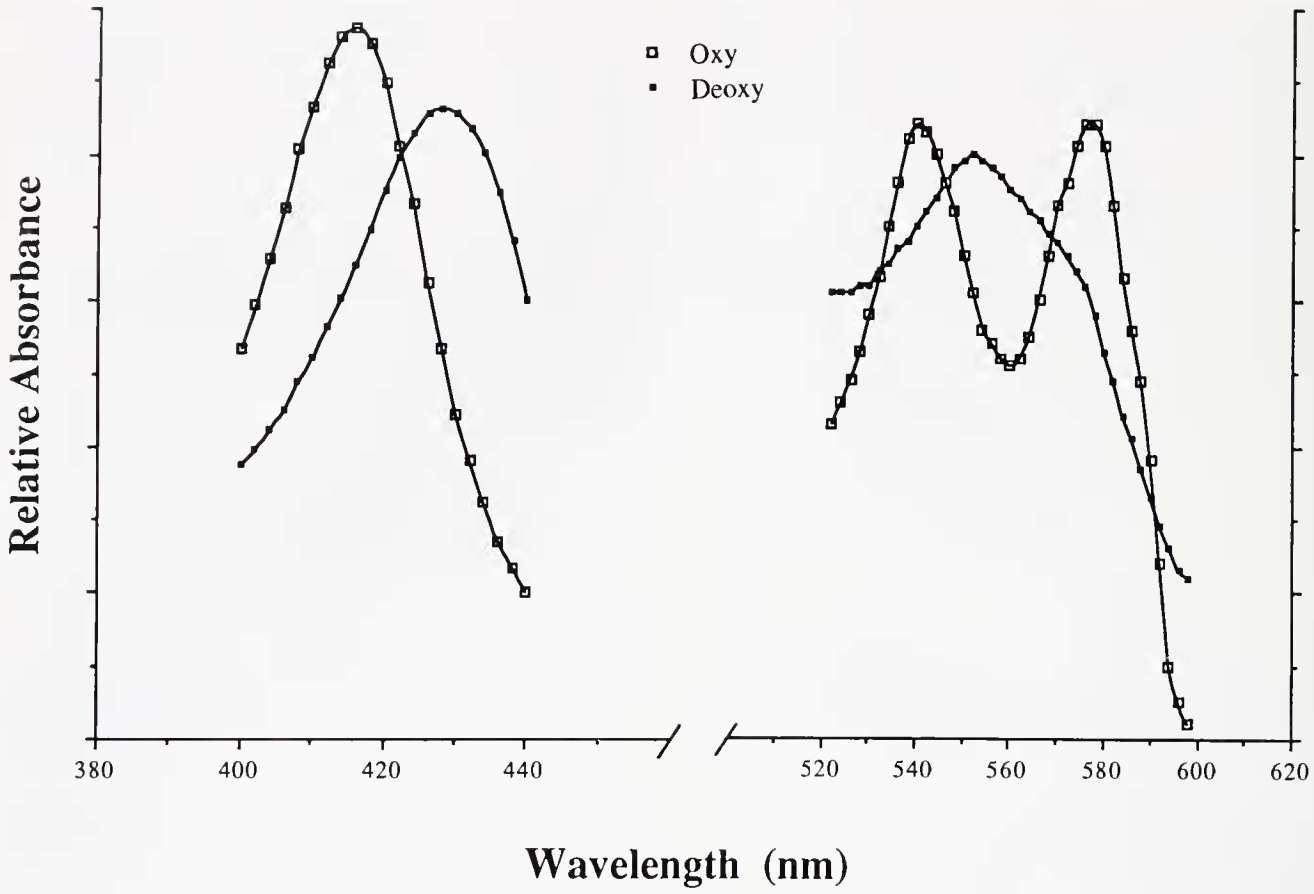

Figure 1. *In vivo* absorption spectra for metacercaria hemoglobin in oxygenated and deoxygenated states.

measured pH values due to rapid loss of carbon dioxide will be toward more basic pH's.

High Performance Liquid Chromatography (HPLC) gel filtration using a LDC Milton Roy CCM HPLC Automation system was performed on metacercaria hemoglobin, host HMW hemoglobin, and host coelomic cell hemoglobin samples to compare their approximate molecular sizes. Samples containing 10–50 μg/ml protein in 100 μl were eluted on a TSK-30 column, 7.5 × 300 mm (Bio-Rad), equilibrated with 0.05 *M* Tris, pH 7.4, at a flow rate of one ml/min and 500–1000 psi. Elution fractions as determined at 415 nm, an absorption maximum for heme proteins, were collected and stored at −80°C for later analysis. Metacercaria hemoglobin was also chromatographed on a 1 × 25 cm Sephacryl-400 column, equilibrated with the same buffer, to more closely determine the molecular weight of the high molecular weight fraction.

HPLC anion exchange chromatography was performed on metacercaria, *Amphitrite* HMW, and coelomic cell hemoglobins, and on the elution fractions from gel filtration experiments. Hemoglobin fractions were determined by detection at 415 nm. Separation was achieved using a TSK-DEAE-5-PW column, 7.5 × 75 mm (Bio-Rad), initially equilibrated with buffer A (0.01 *M* Tris, pH 9.0). A linear salt gradient was established from 0% to 50% buffer B over a 50 minute run time. Buffer B was 0.01 *M* Tris, pH 9.0, with 1.0 *M* sodium acetate. All reagents used were HPLC grade.

Incorporation of H^3-glycine into proteins by metacercariae was determined by incubating 250 animals for 24 hours at 16°C in 2.0 ml of 0.1 m*M* glycine (specific activity of 10 μCi/ml) in 0.05 *M* Tris buffered seawater, pH 7.4. Control animals were incubated in an identical buffer with unlabeled glycine for 24 hours at 16°C. All animals had been starved outside of the host for 12 hours prior to the start of the experiment. Following incubation, controls and experimentals were washed four times with 50 m*M* Tris buffered seawater, pH 7.4, at 0°C and then placed in 250 to 500 μl of 50 m*M* Tris buffer, pH 7.4. Samples stored at −80°C were thawed, sonicated for 15 seconds, centrifuged at 16,000 × *g* for 2 min to remove cell debris, and placed on ice. HPLC gel filtration was run on 100 μl volumes of control and experimental

Table I

Comparison of absorption maxima (nm) for metacercaria, Amphitrite ornata *and human hemoglobins*[△]

		Amphitrite ornata			
		Vascular	Coelomic	Metacercaria	Human
OXY	α	576.0 (0.8/8)	577.7 (0.5/5)	578.2 (0.6/13)	577.4 (0.5/8)
	β	541.1 (0.4/8)	540.9 (0.9/5)	541.3 (0.7/13)	542.5 (0.8/8)
	γ	418.4 (0.7/8)	416.1 (0.4/2)	415.7 (0.9/13)	415.4 (0.5/8)
DEOXY	αβ	554.7 (2.7/6)	555.5 (1.0/4)	559.9 (2.1/7)	555.4 (0.8/8)
	γ	430.2 (0.8/6)	431.5 (−/1)	428.5 (1.6/7)	430.8 (0.5/8)
CARBOXY	α	569**	570**	570.4 (2.9/2)	568.5*
	β	538**	538**	538.9 (0.4/2)	539*
	γ	421**	421**	420.6 (0.4/2)	420*

* Data are from Van Assendelft (1970).
** Data are from Chiancone *et al.* (1980).
△ The numbers in parentheses represent standard deviation/number of samples.

samples (see above for conditions). Sequential one-half ml fractions of the entire gel filtration eluent were collected and the amount of label in each determined using a Beckman LS7000 Liquid Scintillation Counter.

Results

In vivo spectrophotometry of metacercaria tissues showed characteristic hemoglobin spectra when the tissues were exposed to oxygen, nitrogen, and carbon monoxide. The *in vivo* absorption spectra for metacercaria hemoglobin are shown in Figure 1. The absorption maxima of metacercaria tissue hemoglobin and *Amphitrite ornata* HMW and coelomic cell hemoglobins are listed in Table I along with the maxima for human hemoglobin. Only minor variations in maxima are apparent between all four pigments. Absorption spectra of metacercaria gut contents gave absorption profiles that were not recognizable as typical for hemoglobin in oxy, deoxy, or met state (Van Assendelft, 1970).

Oxygen equilibrium curves for metacercaria hemoglobin *in vivo* are shown in Figure 2. The *in vivo* oxygen binding properties of metacercaria hemoglobin are summarized in Table II. The hemoglobin has a high affinity for oxygen and demonstrates cooperative oxygen binding. It exhibits a positive Bohr effect between pH 6.8 and 7.4 (−0.35). There is no significant Bohr effect between pH 7.4 and 7.8. The pH of *Amphitrite* coelomic fluid directly removed from the animal was found to range from 6.9 to 7.6, with a mean pH of 7.3 (SD = 0.2, n = 10). Following aeration of the coelomic fluid, the pH ranged from 7.3 to 7.8, with a mean pH of 7.5 (SD = 0.1, n = 10). The functional properties of metacercaria tissue hemoglobin are compared with *in vitro* oxygen binding characteristics of *Amphitrite* HMW and coelomic cell hemoglobins in Table III. The P_{50} (the O_2 partial pressure required to achieve half-saturation) of metacercaria hemoglobin *in vivo* is lower than either of the *Amphitrite* hemoglobins *in vitro*. Values for host vascular hemoglobin are similar to those reported by Mangum *et al.* (1975) and Chiancone *et al.* (1981). Our measurements of *in cellulo* P_{50} for *Amphitrite* coelomic cell hemoglobin gave slightly lower affinity (higher P_{50}) than the *in vitro* and *in vivo* (cell suspension) values listed in Table III from Mangum *et al.* (1975). Metacercaria tissue hemoglobin shows greater cooperativity than either hemoglobin from *Amphitrite*, and has a significant Bohr effect unlike the host hemoglobins.

Cross sections of the gut ceca of metacercariae showed the presence of *Amphitrite* coelomocytes. Other cells and debris were present which had the coloration and appear-

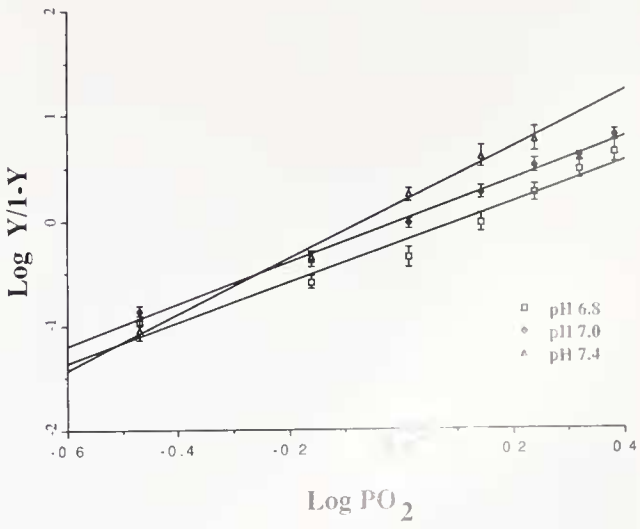

Figure 2. *In vivo* oxygen equilibrium curves for metacercaria hemoglobin. Vertical bars show standard error.

Table II

Oxygen binding characteristics of metacercaria hemoglobin

pH	P_{50} (mmHg)	Hill constant	n
6.8	1.32 ± 0.39*	2.25 ± 0.29	8
7.0	1.12 ± 0.29	2.22 ± 0.24	10
7.4	0.81 ± 0.22	3.06 ± 0.40	7
7.8	0.78 ± 0.23	2.39 ± 0.38	6

* S.D.

ance of the highly vascularized metanephridial tissue; however, transmission electron micrographs of gut ceca and gut lining cells revealed no evidence of material that resembled the characteristic annelid high molecular weight extracellular hemoglobins.

Figure 3 shows the elution profile of metacercaria hemoglobin from HPLC gel filtration which separates molecules on the basis of their size. Two fractions were eluted; fraction A eluted in the void volume of the column (MW > 500,000 daltons) and fraction B eluted at 16,000 daltons. Standard gel filtration on a Sephacryl S-400 column (Fig. 4) indicated that fraction A has a molecular weight of $2.5–3 \times 10^6$ daltons.

HPLC ion exchange chromatograms of whole metacercaria lysate and gel filtration fractions of metacercaria hemoglobin are shown in Figure 5a–c. Ion exchange separation of whole metacercaria hemoglobin resulted in four distinct protein components (Fig. 5a). Metacercaria gel filtration fraction A (MW ~ $2.5–3 \times 10^6$ daltons) when chromatographed on the ion exchange column demonstrated one broad peak with a distinct shoulder,

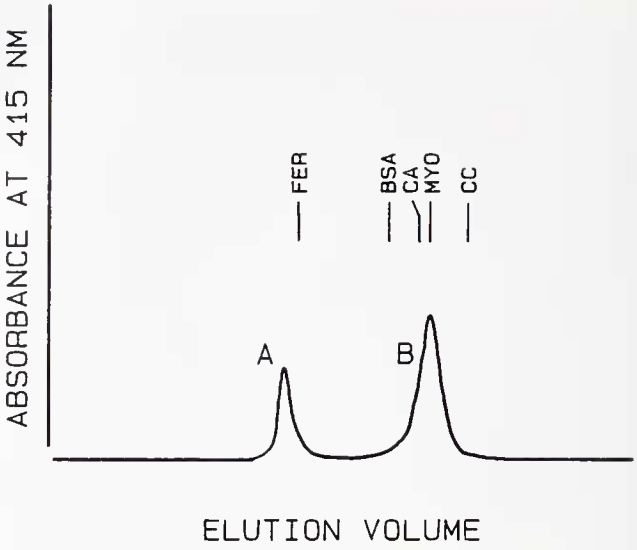

Figure 3. High performance liquid chromatography (HPLC) of metacercaria whole animal lysate. Key to standards: FER, ferritin; BSA, bovine serum albumin; CA, carbonic anhydrase; MYO, myoglobin; CC, cytochrome C.

indicating the presence of two components that were incompletely separated (Fig. 5b). These components of fraction A probably correspond to protein components I and II of whole metacercaria hemoglobin (Fig. 5a). The ion exchange chromatogram of metacercaria gel filtration fraction B (MW ~ 16,000 daltons) is shown in Figure 5c. It demonstrated two peaks that probably correspond to components III and IV on the whole metacercaria hemoglobin chromatogram (Fig. 5a).

Ion exchange chromatograms of *Amphitrite ornata* vascular HMW hemoglobin and coelomic cell hemoglobin are shown in Figure 5d–e. The ion exchange chromatograms of metacercaria whole hemoglobin (Fig. 5a) and the high molecular weight fraction of metacercaria hemoglobin (Fig. 5b) when compared with the elution profile of *Amphitrite* vascular hemoglobin (Fig. 5d) show no correspondence of any component. The ion exchange elution profile of *Amphitrite* coelomic cell hemoglobin (Fig. 5e) shows three protein components, all of which are distinct from the protein components that compose metacercaria whole hemoglobin (Fig. 5a) and the components of the 16,000 MW fraction of metacercaria hemoglobin (Fig. 5c).

Incorporation of H^3-glycine into proteins by these metacercariae was demonstrated. Figure 6 shows a gel filtration elution profile for metacercaria proteins compared to a histogram representing total CPM (background and label) recorded from sequential 1/2 ml elution fractions. The cross-striated portions of the absorbance profile indicate the location of hemoglobin fractions. These results demonstrate the presence of sig-

Table III

Comparison of oxygen binding characteristics of metacercaria and Amphitrite ornata *hemoglobins*

	P_{50} (mm Hg)	Hill constant	Bohr coefficient
A ornata coelomic cell			
in cellulo (pH 7.4)	3.35 (SD, 0.38)	1.20 (SD, 0.19)	—
cell suspension* (pH 6.8–7.8)	1.56–2.60	0.811–1.117	—
*in vitro** (pH 6.25–9.05)	2.72	0.91	NS
vascular* (pH 5.81–7.97)	10.00	1.286	NS
vascular** (pH 6.0–7.4)	6.3–10	≅1.5	—
Metacercaria (pH 6.8–7.4)	0.81–1.32	2.22–3.06	−0.35

* Data is from Mangum *et al.* (1975).
** Data is from Figure 3 of Chiancone *et al.* (1981).

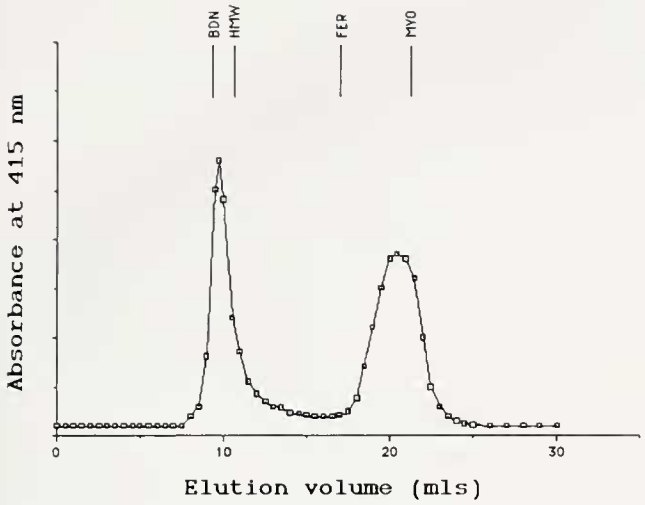

Figure 4. Sephacryl-400 chromatography of metacercaria whole animal lysate. Key to standards: BDN, blue dextran; HMW, *Amphitrite* HMW vascular hemoglobin; FER, ferritin; MYO, myoglobin.

nificant amounts of tritiated glycine in eluted fractions containing proteins which include hemoglobins.

Discussion

The absorption spectra of the pigment in metacercaria tissues identifies it as a heme protein. The pigment appears to be in all body cells but may also be located in intercellular spaces as in some entosymbiotic rhabdocoel flatworms (Jennings and Cannon, 1985). The metacercaria hemoglobin exhibited reversible spectral shifts when alternately exposed to oxygen and nitrogen, indicating a functional ability to bind oxygen. The absorption maxima for oxy, deoxy and carboxy forms of the hemoglobin closely resemble other functional hemoproteins, including both host hemoglobins (Weber *et al.*, 1977). Cain (1969a) demonstrated the presence of heme protein in several larval trematode stages by positive benzidine reaction. However, this is the first larval trematode shown to possess a hemoglobin capable of functional oxygen transport.

Hemoglobin-containing adult trematode, monogenean, nematode, and arthropod parasites are known from many vertebrate and invertebrate hosts (Lee and Smith, 1965; Thurston, 1970; Von Brand, 1973). Many of these parasites live in regions in the host that have low PO_2 such as the liver or the lumen of the gut. Some, like the nematode *Syngamus trachea* (Rose and Kaplan, 1972), live in high PO_2 environments. Hemoglobin-containing turbellarian worms of the families Pterastericolidae and Umagillidae have been identified living in the guts of their echinoderm hosts (Jennings and Cannon, 1985, 1987). As in other animals, hemoglobin in parasitic or endocommensal animals is presumed to function in facilitated diffusion, transport, or storage of oxygen in low PO_2 environments (Weber, 1980; Sharpe and Lee, 1981). Very little is known, however, about the actual function of hemoglobin in these symbiotes.

In general, hemoglobins in symbiotic helminths have high affinities for oxygen (Lee and Smith, 1965; Von Brand, 1973). Host hemoglobins, when present, have lower affinities than the symbiote hemoglobins. The high affinity symbiote hemoglobins may allow the symbiotes to extract oxygen from low PO_2 environments. In some parasitic nematodes such as *Ascaris* and *Strongylus*, the hemocoel hemoglobin affinities are so high that it is doubtful that these hemoglobins function in aerobic metabolism (Okazaki and Wittenberg, 1965). However, if a symbiote's hemoglobin unloads oxygen in the range of oxygen tensions encountered in the host tissues, it is likely to serve a respiratory function.

The gymnophallid metacercariae living inside the host metanephridia are separated physically from the host's coelomic cavity by the thin, highly vascularized walls of the metanephridia. During high tide with normal ventilation, transport of O_2 by HMW vascular hemoglobin in *Amphitrite* probably controls the PO_2 within both the metanephridia and the coelom. Mangum *et al.* (1975) determined that under these conditions, coelomic PO_2 would be close to the P_{50} of the HMW vascular hemoglobin ($\sim$10 mm Hg at physiological pH). At low external PO_2, as would be experienced during low tide, the vascular HMW hemoglobin fails to load oxygen at the gills (Mangum *et al.*, 1975). Thus, the primary supply for oxygen to the metanephridia and metacercariae is interrupted. At this time, the source of oxygen for the metanephridia and metacercariae is the coelomic fluid. The PO_2 inside the metanephridia probably declines below coelomic PO_2 (near coelomic cell hemoglobin P_{50} of 2.7 mm Hg) due to a combination of oxygen consumption by the metanephridial lining and consumption by the metacercariae which can occupy as much as 50% or more of the nephridial space. During these periods of hypoxia, the high affinity hemoglobin of the metacercariae (P_{50} = 1.2 mm Hg) may allow them to obtain oxygen at greater rates by facilitated diffusion. Under anoxic conditions, it may allow them to extend aerobic metabolism by acting as a short term store.

Amphitrite coelomocytes that are swept into the metanephridia through the nephrostome are observed in the gut of the larvae. Although the metanephridial lining is highly vascularized and at most only one cell layer separates the host vascular hemoglobin from the trematode larvae, we were unable to demonstrate ingestion of host HMW hemoglobin by metacercariae. However, if metacercariae are feeding on metanephridial tissue as is suggested by the appearance of similar material in their gut

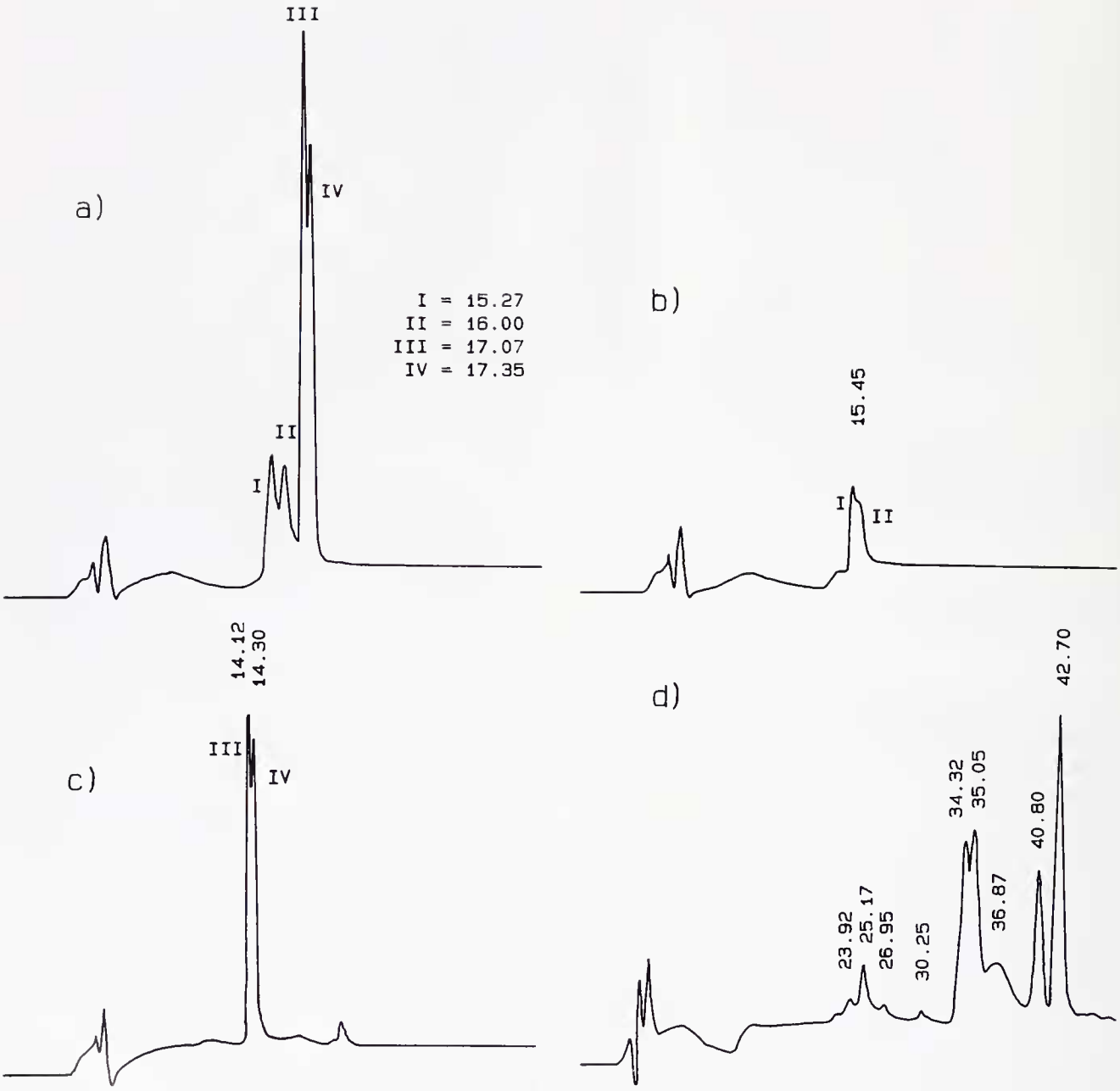

Figure 5. HPLC anion exchange chromatography of a) metacercaria whole animal lysate, b) metacercaria gel filtration fraction A*, c) metacercaria gel filtration fraction B*, d) *Amphitrite ornata* HMW vascular hemoglobin, and e) *Amphitrite ornata* coelomic cell hemoglobin. Chromatograms are not to scale. Absorbance monitored at 415 nm. Decimal numbers represent elution times.

* These samples were loaded onto the ion exchange column in gel filtration buffer which had a different ionic strength from the other samples which were at the ionic strength of seawater. Therefore, elution times will not compare.

ceca, then it is probable that ingestion of host vascular hemoglobin might also occur. Ingestion of host hemoglobin as a source of nutrition is a common occurrence among vertebrate parasites (Halton, 1967; Schmidt and Roberts, 1985) but until now has not been observed among parasites of invertebrates.

Halton (1974) showed that hemoglobin ingested by the monogenean *Diclidophora merlangi* is taken up directly into cecal cells by pinocytosis and then broken down. If gut lining cells of the metacercaria larvae took up intact *Amphitrite* hemoglobins by pinocytosis as does *Diclidophora*, it is possible that these hemoglobins may

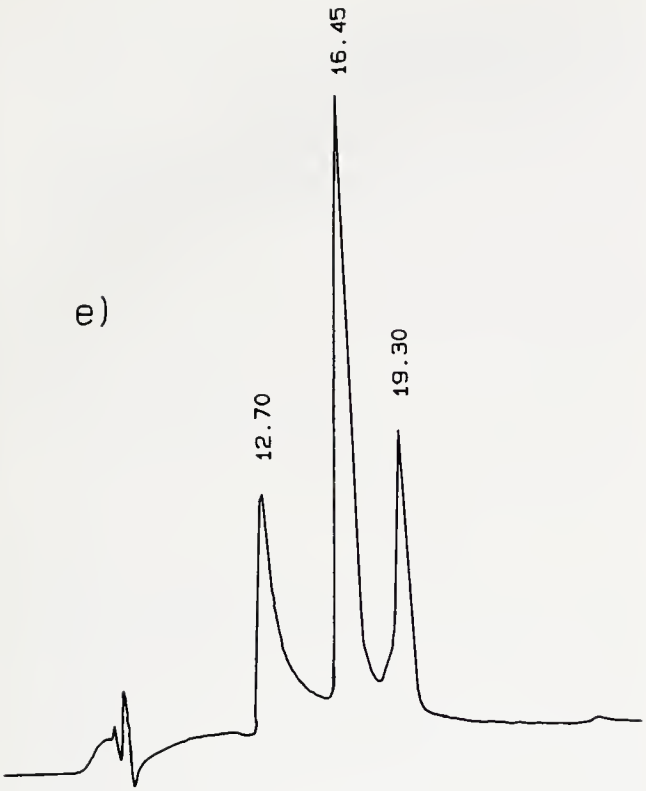

Figure 5. Continued.

not be completely degraded or not broken down at all, but rather retained and functionally altered.

The metacercariae possess a monomeric hemoglobin with an approximate molecular weight of 16,000 and two different globin forms. This corresponds to the results found for the trematodes *Fasciola gigantica* (Lutz and Siddiqi, 1967), *Philophthalmus megalurus,* and *Echinostoma revolutum* (Cain, 1969a), each of which possess two globin forms of hemoglobin with molecular weights ranging from 15 to 17,000 daltons. The two globins found in metacercaria tissues are distinct from the globins of *A. ornata* coelomic cell hemoglobins based on spectral differences and on elution differences using ion exchange media.

Tritiated glycine was found in gel filtration fractions that contain metacercaria hemoglobins. This evidence, along with ion exchange elution differences, spectral and functional differences, strongly suggests that at least the globin portion of the metacercaria hemoglobin is produced in the animal. Because glycine is also used in the synthesis of the protoporphyrin ring (Guyton, 1986), the appearance of label in metacercaria hemoglobin may also indicate the *de novo* synthesis of heme. Alternatively, heme groups could be obtained directly from ingested host hemoglobins, making synthesis unnecessary. Cain (1969b) showed that *Fasciolopsis buski* did not produce heme, but did synthesize the globin portion of its hemoglobin. This species also ingests its host's hemoglobin and, therefore, has an exogenous supply of heme. On the basis of the available evidence, the origin of heme in the metacercaria hemoglobin is unresolved.

The metacercariae also have a large molecular weight heme protein fraction. This fraction elutes as two distinct peaks upon ion exchange chromatography that correspond to two consistent hemoglobin peaks present in the whole metacercaria lysate. These two heme proteins demonstrate the presence of radiolabel and are chromatographically distinct from all hemoglobin fractions of *Amphitrite* HMW hemoglobin, indicating that they were synthesized in the larvae. *Ascaris* possesses two hemoglobin fractions, one low molecular weight form found in the tissues and a high molecular weight aggregate in the perienteric fluid (Okazaki *et al.*, 1965, 1967). However, there have been no reports of a high molecular weight hemoglobin in a trematode. We are uncertain at this time whether the heme proteins of the metacercaria high molecular weight fraction are functional hemoglobins. It may be that the two heme protein peaks seen in

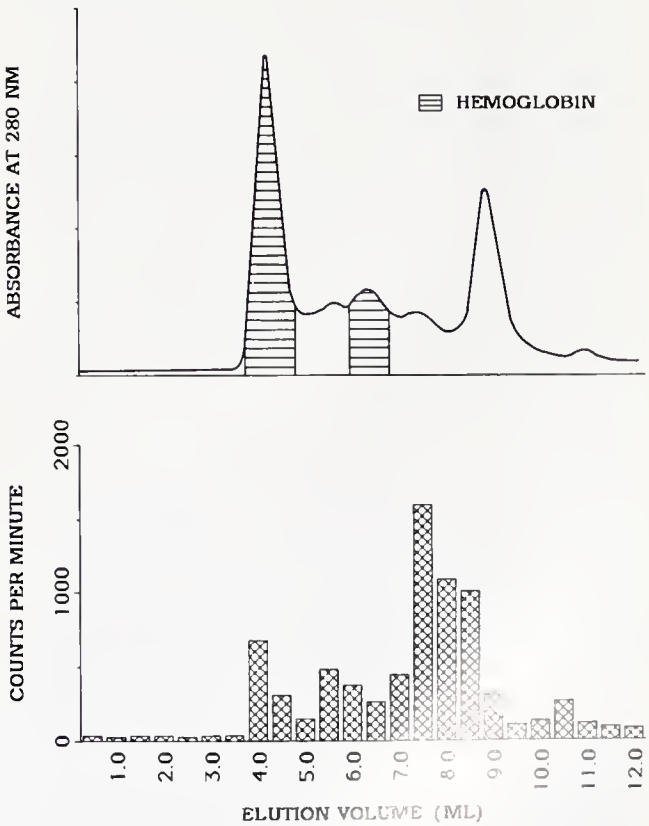

Figure 6. HPLC gel filtration elution profile for proteins from lysates of whole metacercaria incubated in H^3-glycine compared to a histogram of radiolabel (CPM) in sequential one-half ml samples of eluent.

the high molecular weight fraction are aggregations of altered forms of the two low molecular weight hemoglobins. Further research will be needed to determine the exact nature of this heme protein fraction from these animals.

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